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False-negative result in molecular diagnosis of SARS-CoV-2 in samples with amplification inhibitors

Resultado falso negativo no diagnóstico molecular de SARS-CoV-2 em amostras com inibidores de amplificação

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ABSTRACT

Introduction: Although reverse transcription-polymerase chain reaction (rRT-PCR) is the gold standard method for detecting severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), some factors, such as the presence of amplification inhibitors, lead to false-negative results. **Objective:** Here we describe the differences between rRT-PCR results for SARS-CoV-2 infection in normal and diluted samples, simulating the need for dilution due to the presence of amplification inhibitors. **Material and method:** Viral ribonucleic acid (RNA) from samples of nasopharyngeal swabs from 20 patients previously detected as "Negative" and 21 patients detected as "Positive" for SARS-CoV-2 was performed with the EasyExtract DNA-RNA kit (Interprise®). The rRT-PCR was performed with the OneStep/COVID-19 kit (IBMP), with normal and diluted (80 µl of H_2O RNAse free) samples, totaling 82 tests. **Results:** The results indicate that there is an average variation ($\alpha < 0.05$) delaying the Cq between the results of amplification of the internal control (IC), *N* gene (*N*G), and ORF1ab (OF), 1.811 Cq, 3.840 Cq, and 3.842 Cq, respectively. **Discussion:** The extraction kit does not completely purify the inhibitor compounds; therefore, no amplified product result may occur. In this study, we obtained a 19.04% false-negative diagnosis after sample dilution; this process reduces the efficiency of rRT-PCR to 29.8% in detecting SARS-CoV-2. **Conclusion:** Knowing the rRT-PCR standards of diluted samples can assist in the identification of false-negative cases and, consequently, avoid incorrect diagnosis.

Key words: COVID-19; rRT-PCR; dilution; viral diagnosis; RNA extraction.

RESUMO

Introdução: *Embora a reação em cadeia da polimerase de transcrição reversa (rRT-PCR) seja o método padrão-ouro para detecção de coronavírus da síndrome respiratória aguda grave 2 (SARS-CoV-2), alguns fatores, como a presença de inibidores de amplificação, levam a resultados falso negativos.* ***Objetivo:*** *Descrevemos as diferenças entre os resultados de rRT-PCR para infecção por SARS-CoV-2 em amostras normais e diluídas, simulando a necessidade de diluição devido à presença de inibidores de amplificação.* ***Material e método:*** *A extração de ácido ribonucleico (RNA) viral de amostras de suabes nasofaríngeos de 20 pacientes previamente detectados como "negativos" e 21 pacientes detectados como "positivos" para SARS-CoV-2 foi realizada com* kit *o EasyExtract DNA-RNA (Interprise®). A rRT-PCR foi realizada com o* kit *OneStep/COVID-19 (IBMP), com amostras normais e diluídas (80 µl de H_2O RNAse-free), totalizando 82 testes.* ***Resultados:*** *Os resultados indicam que existe uma variação média ($\alpha < 0,05$) atrasando o Cq entre os resultados de amplificação do controle interno (CI), gene* N *(G*N*) e ORF1ab (OF) de 1,811 Cq, 3,840 Cq e 3,842 Cq, respectivamente.* ***Discussão:*** *O* kit *de extração não purifica completamente os compostos inibidores, portanto, pode ocorrer não amplificação. Obtivemos um diagnóstico falso negativo de 19,04% após a diluição da amostra; esse processo reduz a eficiência da rRT-PCR para 29,8% na detecção de SARS-CoV-2.* ***Conclusão:*** *Conhecer os padrões da rRT-PCR de amostras diluídas pode auxiliar na identificação de casos falso negativos e, consequentemente, evitar um diagnóstico incorreto.*

Unitermos: *COVID-19; rRT-PCR; diluição; diagnóstico viral; extração de RNA.*

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RESUMEN

Introducción: *Aunque la reacción en cadena de la polimerasa con transcriptasa reversa en tiempo real (rRT-PCR) sea el método de referencia para detección del coronavirus tipo 2 del síndrome respiratorio agudo grave (SARS-CoV-2), algunos factores como la presencia de inhibidores de amplificación conducen a resultados falsos negativos.* ***Objetivo:*** *Describimos las diferencias entre los resultados de rRT-PCR para infección por SARS-CoV-2 en muestras normales y diluidas, simulando la necesidad de dilución debido a la presencia de inhibidores de amplificación.* ***Material y método:*** *La extracción de ácido ribonucleico (ARN) viral de muestras de hisopos nasofaríngeos de 20 pacientes previamente detectados como "negativos" y 21 pacientes detectados como "positivos" para SARS-CoV-2 se realizó con el kit Easy Extract DNA-RNA (Interprise®). La rRT-PCR se realizó con el kit OneStep/COVID-19 (IBMP), con muestras normales y diluidas (80 µl de H_2O libre de ARNasa), totalizando 82 pruebas.* ***Resultados:*** *Los resultados indican que hay una variación media ($\alpha < 0,05$) retrasando el ciclo de cuantificación (Cq) entre los resultados de amplificación del control interno (CI), gen N (GN) y ORF1ab (OF) de 1,811 Cq, 3,840 Cq y 3,842 Cq.* ***Discusión:*** *El kit de extracción no purifica completamente los compuestos inhibidores; por lo tanto, puede ocurrir no amplificación. Obtuvimos un diagnóstico falso negativo de 19,04% después de la dilución de la muestra; ese proceso reduce la eficiencia de la rRT-PCR hacia 29,8% en la detección de SARS-CoV-2.* ***Conclusión:*** *Conocer los patrones de la rRT-PCR de muestras diluidas puede ayudar en la identificación de casos falsos negativos y, por consiguiente, evitar un diagnóstico equivocado.*

Palabras clave: *COVID-19; rRT-PCR; dilución; diagnóstico virológico; extracción de ARN.*

INTRODUCTION

The first confirmed case of Coronavirus disease 2019 (COVID-19) in Latin America occurred in Brazil, on February 25, 2020[1]. Since then, until August 2020, Brazil has recorded about 4.1 million cases and about 126 thousand deaths due to COVID-19[2].

Early detection of infected individuals with large-scale testing, immediate isolation of screened cases, preventive self-isolation of close contacts, and prompt treatment for severe cases are essential measures to reduce the spread of the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)[3].

Therefore, to quickly diagnose infections and mitigate the transmission of SARS-CoV-2, the real-time reverse transcription-polymerase chain reaction (rRT-PCR) is being used as the primary method in research and hospital laboratories to identify the virus in respiratory samples such as sputum or nasal, throat, nasopharyngeal swabs[4].

The rRT-PCR tests typically take 4 to 6 hours to complete, with extraction, amplification, and detection of ribonucleic acid (RNA)[5]. Considering the limited supply of extraction reagents and test kits worldwide, extraction kits without RNA purification aim to solve this limitation and shorten the extraction time, thereby shortening the response time[4, 5].

However, amplification inhibitors, organic and inorganic substances, may be present in the original samples or be introduced during samples transportation, processing, or RNA extraction, causing partial amplification inhibition, leading to a decreased PCR sensitivity or total inhibition and, consequently, to false-negative results[6].

Extraction kits without RNA purification may need to optimize rRT-PCR by sample dilution in case of problems with the rRT-PCR amplification, thus minimizing the presence of amplification inhibitors[7], allowing amplification even in the presence of inhibitors or some sample degradation, avoiding the need to request a new sample from the patient, however, it is necessary to know the diluted amplification patterns, avoiding false-negative diagnosis.

Due to the severity of the pandemic, test kits were and are being developed and approved quickly to meet the worldwide demand for large-scale tests, generating the need for information on real data on the use of these kits in diagnostic laboratories[8]. Here we describe the differences between the rRT-PCR results for SARS-CoV-2 infection in normal and diluted samples, simulating the need for dilution due to the presence of amplification inhibitors.

MATERIAL AND METHOD

Nasopharyngeal swab samples of RNA extraction

Samples of nasopharyngeal swabs from 41 patients admitted to the Ministro Costa Cavalcanti Hospital in Foz do Iguaçu, Paraná state, Brazil, were selected. Twenty of these patients were previously detected as "negative" and 21 patients were detected as "positive"

for SARS-CoV-2 infection by the rRT-PCR diagnosis. The swabs were stored in tubes with 1× phosphate-buffered saline (PBS 1×), at -20ºC, until extraction.

The EasyExtract deoxyribonucleic acid (DNA)-RNA kit (Interprise®), lot ITBR0720, was validated by comparing the results found using the Applied Biosystems™ MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit (Thermo Fisher Scientific®), lot 200312, at 1% significance level (n = 96). For the viral RNA extraction, 20 µl of the EasyExtract DNA-RNA (Interprise®) reagent was mixed with 20 µl of PBS 1× from the swab samples in 1.5 ml Eppendorf tubes. The tubes were shaken in a vortex mixer (Kasvi, K45-2810) at 1.050 rpm for 15 seconds, incubated at 95ºC for 5 minutes, and refrigerated at -20ºC for RNA stabilization[7].

Samples dilution and rRT-PCR for SARS-CoV-2

The samples were diluted in 80 µl of UltraPure® H_2O RNAse free (1:2), totaling 82 tests (41 normal samples and 41 diluted samples).

The rRT-PCR assay was performed using the Biomol OneStep/COVID-19™ Kit (IBMP), lot 200399Z074, 15 µl of reaction rRT-PCR Mix and 5 µl of purified sample RNA (from RNA extraction) or purified negative control, were pipette up and down to mix and, for positive control, 15 µl of the Mix reaction was mixed with 5 µl of the positive control[9].

The analysis was performed using the QuantStudio™ 5 Real-Time PCR Systems equipment (Thermo Fisher Scientific®), under the conditions: i) hold stage – 50ºC for 15 minutes (one cycle), 95ºC for 3 minutes (one cycle); ii) PCR stage – 95ºC for 15 seconds and 55ºC for 40 seconds (40 cycles); iii) hold stage – 25ºC for 10 seconds (one cycle). The threshold values of the internal control (ROX), ORF1ab (FAM), and gene *N* (HEX/VIC) were 20,000, 30,000, and 40,000, respectively, with a baseline from 5 to 15, according to the IBMP protocol[9].

The results were evaluated by the rRT-PCR amplification standards, amplification values and submitted to descriptive analysis, normality test, and analysis of variance (Anova), to detect differences between the results before and after dilution.

The efficiency of the rRT-PCR

The analytical efficiencies for detecting SARS-CoV-2 from the normal methodology and after dilution were performed by serial dilution in the following proportions: 1, 1:2, 1:4, 1:8, and 1:10 (view Efficiency Curves in supplementary files). The results were evaluated by scatter plots and the efficiency values calculated from the R^2 of the linear regression (view Efficiency Test in supplementary files).

RESULTS

The results are described in **Table**. Comparisons between the results of the diluted and undiluted sample indicate that there is an average variation ($\alpha < 0.05$) delaying Cq between the results of amplification of the internal control (IC), *N* gene (*N*G), and ORF1ab (OF) 1.811 Cq, 3.840 Cq, and 3.842 Cq, respectively.

The Cq means of the IC were 29.423 for the original samples and 31.280 for the diluted samples; for *N*G, the Cq mean of the original value was 25.816, and 29.848 for diluted samples; for ORF1ab, the average Cq results for samples without dilution were 27.104 against 31.138 for diluted samples.

Before dilution, samples 25, 26, 39, and 40 showed values lower than the cut-off stipulated for ORF1ab, and were considered positive. After dilution, they all shifted the ORF1ab Cq values to the right and were then considered negative due to non-amplification within the cut-off parameters (**Figure 1**).

The normality test considered normal values for IC and *N*G ($\alpha > 0.05$), however, for ORF1ab the values were considered abnormal ($\alpha < 0.05$). When removing outliers, the data returns to normal, indicating that the amplification values of samples 25, 26, 39, and 40 are not within the expected range, indicating a great variation with the other samples ($\alpha > 0.05$).

The amplification efficiency for the normal sample was 99.79% for IC, 99.51% for *N*G, and 97.09% for OF. For the diluted sample, the amplification efficiency was 98.88% for IC, 78.33% for *N*G, and 67.29% for OF, indicating a decrease of 21.18% for *N*G detection and 29.8% for OF detection.

DISCUSSION

The positive control showed amplification for the three targets evaluated in all tests (Cq ≤ 35) and the negative control did not show any amplification for the three evaluated targets, according to the Mix manufacturer's protocol, validating the results.

The tests performed to demonstrate 1:2 dilutions were interesting to obtain a reliable amplification in samples with inhibitors, as shown in **Figure 2**. However, it is important to perceiving the result curve patterns after dilution.

In the example shown in Figure 2A, the sample without dilution did not achieve amplification of the IC (Cq = undetermined value) and OF patterns (Cq = undetermined value), and *N*G (Cq = 29.995) not defined and not showing a perfect exponential

curvature. After dilution (b), the perfect amplification of the three markers is perceived, indicating a superior quality of the sample and the absence of inhibitors. The Cq values of the amplifications were 29.951 for IC, 25.444 for *N*G, and 27.579 for OF.

In cases as in Figure 2, only the dilution is sufficient to diagnose the sample as positive, avoiding new stress for the patient in repeating the collection, and new exposure by the health professional, the infected patient will be referred to the correct

TABLE – Results of the rRT-PCR ΔCq amplification of normal and diluted samples

Diagnosis	Sample ID	IC	IC*	*N*G	*N*G*	OF	OF*	ΔIC	Δ*N*G	ΔOF
Negative	1	26.224	28.376	-	-	-	-	-2.152	-	-
	2	28.941	31.024	-	-	-	-	-2.083	-	-
	3	29.585	31.871	-	-	-	-	-2.286	-	-
	4	29.436	30.428	-	-	-	-	-0.992	-	-
	5	32.186	34.128	-	-	-	-	-1.942	-	-
	6	30.660	32.673	-	-	-	-	-2.013	-	-
	7	27.193	29.721	-	-	-	-	-2.528	-	-
	8	29.451	31.890	-	-	-	-	-2.439	-	-
	9	29.092	31.230	-	-	-	-	-2.138	-	-
	10	25.364	27.492	-	-	-	-	-2.128	-	-
	11	30.608	32.700	-	-	-	-	-2.092	-	-
	12	29.246	31.522	-	-	-	-	-2.276	-	-
	13	30.690	32.037	-	-	-	-	-1.347	-	-
	14	28.590	30.194	-	-	-	-	-1.604	-	-
	15	27.598	28.500	-	-	-	-	-0.902	-	-
	16	27.651	29.382	-	-	-	-	-1.731	-	-
	17	28.691	30.320	-	-	-	-	-1.629	-	-
	18	25.980	27.693	-	-	-	-	-1.713	-	-
	19	28.382	31.124	-	-	-	-	-2.742	-	-
	20	27.813	29.600	-	-	-	-	-1.787	-	-
Positive	21	28.941	30.974	21.011	24.046	19.121	21.320	-2.033	-3.035	-2.199
	22	27.536	29.633	19.276	22.270	24.767	27.180	-2.097	-2.994	-2.413
	23	29.052	31.038	22.909	24.030	27.013	29.706	-1.986	-1.121	-2.693
	24	29.668	31.944	17.969	23.490	25.689	28.211	-2.276	-5.521	-2.522
	25	**29.488**	**32.191**	**21.520**	**35.000**	**28.990**	**40.000**	**-2.703**	**-13.480**	**-11.010**
	26	**30.437**	**32.970**	**20.175**	**32.377**	**28.299**	**40.000**	**-2.533**	**-12.202**	**-11.701**
	27	28.067	29.983	23.868	28.955	24.136	26.759	-1.916	-5.087	-2.623
	28	22.793	23.674	24.278	25.226	25.345	26.093	-0.881	-0.948	-0.748
	29	23.423	25.454	21.491	24.937	21.280	25.490	-2.031	-3.446	-4.210
	30	26.926	28.030	17.773	21.417	17.873	21.780	-1.104	-3.644	-3.907
	31	30.772	30.907	31.018	31.312	29.833	31.767	-0.135	-0.294	-1.934
	32	28.332	30.406	24.273	27.285	23.178	25.684	-2.074	-3.012	-2.506
	33	27.453	28.774	28.912	30.416	27.939	29.197	-1.321	-1.504	-1.258
	34	29.475	30.981	24.052	25.287	23.684	24.918	-1.506	-1.235	-1.234
	35	32.237	32.427	27.702	31.440	27.225	30.644	-0.190	-3.738	-3.419
	36	26.155	28.695	27.684	31.167	27.783	30.702	-2.540	-3.483	-2.919
	37	29.219	31.453	27.088	30.406	26.308	29.036	-2.234	-3.318	-2.728
	38	28.364	30.639	24.162	28.913	24.462	27.117	-2.275	-4.751	-2.655
	39	**31.006**	**32.548**	**32.500**	**35.380**	**32.914**	**40.000**	**-1.542**	**-2.880**	**-7.086**
	40	**31.776**	**32.410**	**34.074**	**35.756**	**32.333**	**40.000**	**-0.634**	**-1.682**	**-7.667**
	41	32.434	34.160	24.577	27.840	23.906	27.165	-1.726	-3.263	-3.259

The variations were calculated considering the values of the original samples as the true Cq.
**samples diluted in 80 µl; IC: internal control Cq;* NG: N *gene Cq; OF: ORF1ab Cq; Δ: Cq variation. In bold are the false-negative samples after dilution.*

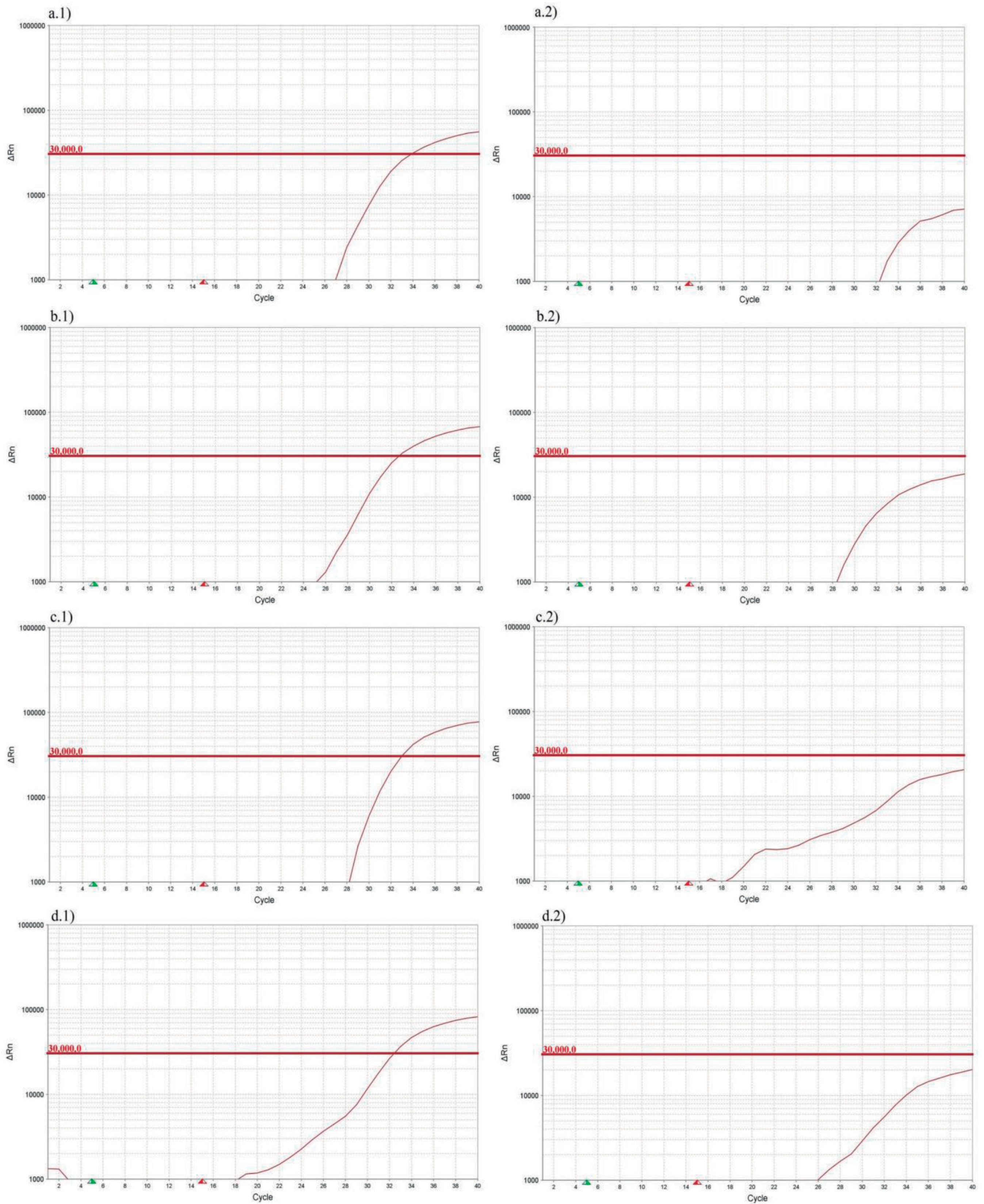


FIGURE 1 – *Amplification of ORF1ab from samples 25 (a), 26 (b), 39 (c) and 40 (d) before (.1) and after (.2) dilution*

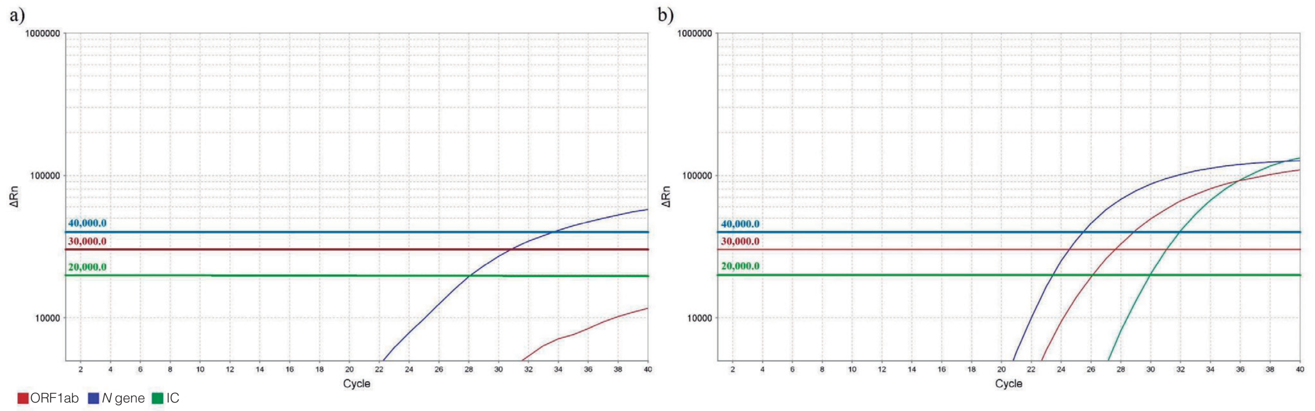
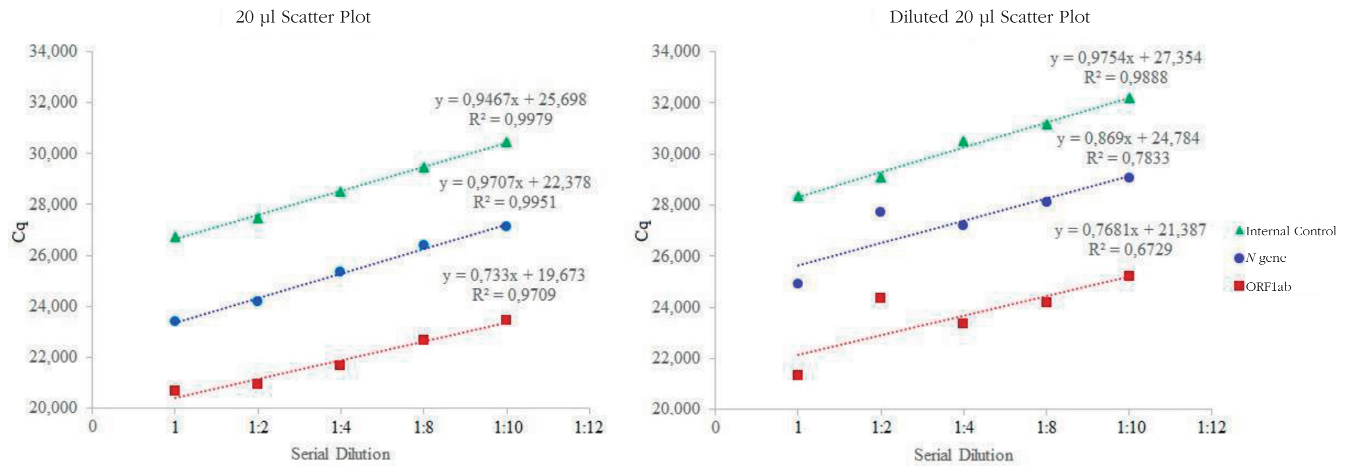
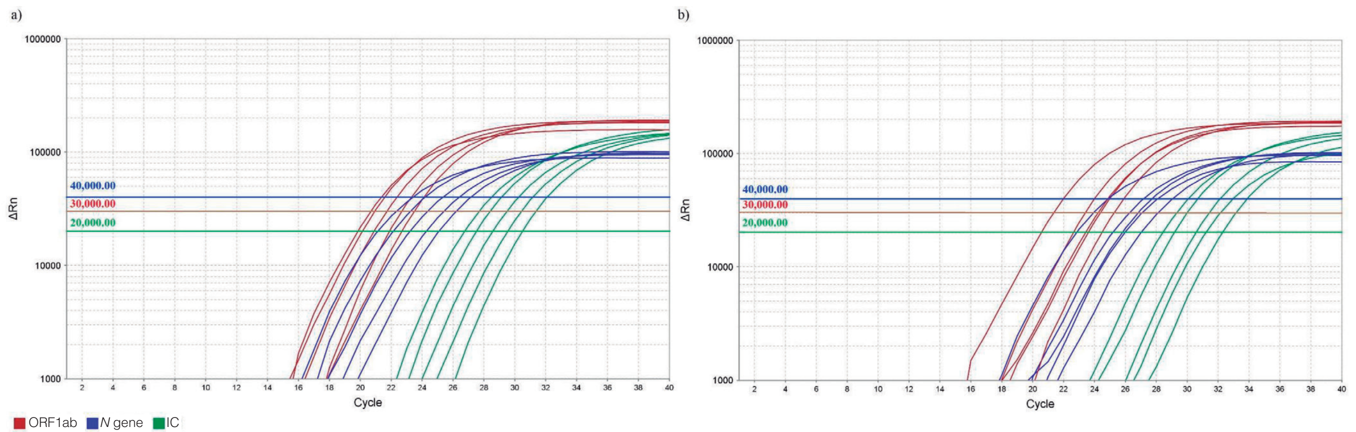


FIGURE 2 – *Differences in amplification patterns of a sample with inhibitors before (a) and after (b) dilution*



Efficiency test



Efficiency curves

treatment site in a short period of time between collection and diagnosis. However, in cases where the result after the dilution is negative, a series of precautions should be taken when releasing the diagnosis, such as the characteristics of the curve, the graph must be evaluated completely and not only the values that exceeded the Cq.

According to the Thermo Fisher® manual, considering a process efficiency of 100%, there is a known variation in Cq when the sample is diluted, which is variable according to the proportion of the dilution. This dilution variation can be $\Delta Cq = 1$ from 1:2, $\Delta Cq = 2$ from 1:4, $\Delta Cq = 3$ to 1:8 and $\Delta Cq = 3.3$ from 1:10. However, these values vary according to the efficiency of the process and presence of inhibitors[10], which can result in false-negative diagnosis in low viral load samples, depending on the value used as a parameter to distinguish between positive and negative.

The positive samples tested that showed divergent results after dilution obtained ΔCq values between 28 and 33, which should not make them negative after dilution even with low efficiency in the amplification process, since there was a variation between 8 and 12 Cq (Figure 1). Considering the progression of ΔCq according to larger dilutions, the dilution proposed by the manufacturer of the viral RNA extraction kit (1:10) would not be interesting, as, theoretically, 1:10 would cause the Cq values to be even later. Larger tests involving smaller dilutions can be performed verifying in what proportion there would be no significant differences in Cq values and the effectiveness in the dilution of the rRT-PCR inhibitors.

The importance of performing rRT-PCR in kits that provide IC marking has already been reported by Kim *et al*. (2016)[10], generating conclusive results about the extraction process, avoiding the release of false-negative results in samples that were not amplified with precision, since the interpretation of the results is not always direct. The sensitivity of rRT-PCR is negatively impacted by compounds present in the clinical sample that may partially or completely inhibit rRT-PCR chemistry[11-15].

Protocols with purification steps can avoid the presence of amplification inhibitors, removing potential endogenous rRT-PCR inhibitors such as detergents, chelating compounds, and guanidine-HCl[11, 13, 16-19]. The efficiency of removing inhibitors from patient samples may be related to the intrinsic properties of the method used to extract the RNA[20], which is not the case of the kit used in this study. The Easy Extract™ kit does not completely purify the inhibitor compounds, which significantly reduces the extraction time; however, non-amplification by inhibitors may occur.

A diagnostic error can lead infected patients to non-COVID-19 areas with the subsequent risk of infection for others areas; or patients who are SARS-CoV-2 negative to be sent to COVID-19 areas[21], generating possible contamination to uninfected patients and also the spread of viruses in the disinfected areas, which can lead to viral spread within hospitals and treatment centers and contaminate health professionals. Knowing the rRT-PCR standards of diluted samples can help in the identification of false-negative cases and, consequently, avoid a wrong diagnosis.

CONCLUSION

The 1:2 dilution of the sample with inhibitors using the UltraPure® H_2O RNAse free generated amplification in 100% of the tested cases, therefore it is an alternative to avoid new sample collection from the patient. However, we emphasize that in this study we obtained 19.04% false-negative diagnosis after sample dilution, and this process reduces the efficiency of rRT-PCR to 29.8% in detecting SARS-CoV-2. It is possible to infer that the dilution helps in cases which a new sample collection is not feasible, but caution is needed in the evaluation of the rRT-PCR result.

It is important to assess the pattern of the amplification curves after dilution to avoid inaccurate diagnosis. If the sample with inhibitors is positive with a high viral load, the result will be reliable if IC and *N*G amplification occur up to Cq 30 and ORF1ab up to Cq 35. In case of non-amplification of the *N*G and ORF1ab curve after dilution, we recommend evaluate the need for a new sample and new analysis.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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